

FULBRIGHT & JAWORSKI L.L.P.

A REGISTERED LIMITED LIABILITY PARTNERSHIP
600 CONGRESS AVENUE, SUITE 2400
AUSTIN, TEXAS 78701-2978
WWW.FULBRIGHT.COM

FACSIMILE TRANSMISSION**DATE:** April 24, 2008**MATTER NUMBER:** 01982 10314516

RECIPIENT(S):	FAX NO.	PHONE NO.:
Attention: Dale Shaw for Judge Donald E. Adams Board of Patent and Appeals and Interferences	571-273-0053	

FROM: David Parker**FLOOR:** 2001**PHONE:** (512) 536-3055**FAX:** (512) 536-4598**RE:** Appeal No. 2007-4320

Serial No. 10/720,987

NUMBER OF PAGES INCLUDING COVER PAGE: 11**MESSAGE:****CAUTION - CONFIDENTIAL**

THE INFORMATION CONTAINED IN THIS FACSIMILE IS CONFIDENTIAL AND MAY ALSO CONTAIN PRIVILEGED ATTORNEY-CLIENT INFORMATION OR WORK PRODUCT. THE INFORMATION IS INTENDED ONLY FOR THE USE OF THE INDIVIDUAL OR ENTITY TO WHOM IT IS ADDRESSED. IF YOU ARE NOT THE INTENDED RECIPIENT, OR THE EMPLOYEE OR AGENT RESPONSIBLE TO DELIVER IT TO THE INTENDED RECIPIENT, YOU ARE HEREBY NOTIFIED THAT ANY USE, DISSEMINATION, DISTRIBUTION OR COPYING OF THIS COMMUNICATION IS STRICTLY PROHIBITED. IF YOU HAVE RECEIVED THE FACSIMILE IN ERROR, PLEASE IMMEDIATELY NOTIFY US BY TELEPHONE, AND RETURN THE ORIGINAL MESSAGE TO US AT THE ADDRESS ABOVE VIA THE U.S. POSTAL SERVICE. THANK YOU.

**IF YOU DO NOT RECEIVE ALL OF THE PAGES, PLEASE CALL
DEBORAH ROSZKOWSKI AT 512-536-3120 AS SOON AS POSSIBLE.**

FULBRIGHT & JAWORSKI L.L.P.

A REGISTERED LIMITED LIABILITY PARTNERSHIP
600 CONGRESS AVENUE, SUITE 2400
AUSTIN, TEXAS 78701-3271
WWW.FULBRIGHT.COM

DAVID L. PARKER
PARTNER
DPARKER@FULBRIGHT.COM

DIRECT DIAL: (512) 536-3055
TELEPHONE: (512) 474-5201
FACSIMILE: (512) 536-4598

April 11, 2008

VIA FACSIMILE - 571-273-0299

Judge Donald E. Adams
Board of Patent Appeals and Interferences
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, Virginia 22313-1450

RE: *Appeal No. 2007-4320 U.S. Patent Application No. 10/720,987 entitled*
"COMPOSITIONS AND SYSTEMS FOR THE REGULATION OF GENES"
by Didier Trono and Maciej Wiznerowicz
Our reference: CLFR:023US

Dear Judge Adams:

During oral argument in the referenced appeal held Tuesday, April 8, 2008, you made the comment, regarding the statement at the bottom of column 1, page 1910 of the Deuschle *et al.* reference ("... many different promoters ... (data not shown)"), that perhaps the authors were holding back information regarding 'other promoters' for publication in a later manuscript. Upon return, I identified the attached reference of Moosmann *et al.*, *Biol. Chem.* 378:669-677 (1997), entitled "Silencing of RNA Polymerase II and III -Dependent Transcription by the KRAB Protein Domain of Kox1, a Krüppel-Type Zinc finger factor." In seeing this (and noting that there was indeed an author in common with the Deuschle *et al.* reference), I thought it important to bring it to the Board's attention.

Very truly yours,

David L. Parker

DLP/ddr
Enclosure

Silencing of RNA Polymerases II and III-Dependent Transcription by the KRAB Protein Domain of KOX1, a Krüppel-Type Zinc Finger Factor

Peter Moosmann¹, Oleg Georgiev¹,
Hans-Jürgen Thiesen², Michael Hagmann¹
and Walter Schaffner^{1,*}

¹ Institut für Molekularbiologie der Universität, Abteilung II,
Winterthurerstr. 190, CH-8057 Zürich, Switzerland

² Institut für Immunologie der Universität Rostock,
Schillingallee 70, D-18055 Rostock, Germany

* Corresponding author

The so-called KRAB domain, which is present in about one third of the vertebrate Krüppel-type zinc finger factors, has previously been shown to inhibit transcription *in cis* when tethered to promoter regions. Here we analyze this effect with fusions of the KRAB domain derived from KOX1/ZNF10 zinc finger protein to the heterologous DNA binding domains of both LexA and GAL4 factors. In transfected human cells, repression of reporter gene transcription is observed not only from proximal promoter positions, but also when KRAB is tethered to DNA at a remote position more than 1.8 kb downstream of the initiation site of transcription. Furthermore, KRAB-mediated silencing over short and long distances is not restricted to RNA polymerase II, since transcription by RNA polymerase III is also repressed. However, transcription by RNA polymerase I and by phage T7 RNA polymerase in mammalian cells are not significantly influenced by the KRAB domain. These latter results may indicate that repression by the KRAB domain, at least under our assay conditions, involves specific inhibition of some component(s) of RNA polymerase II and III transcription, rather than inducing some gross physical alteration of template chromatin structure.

Keywords: Adenovirus VA RNA I / Phage RNA polymerase / Remote gene control / rRNA gene promoter / Silencer / Transcription repression.

Introduction

In all organisms studied so far, gene transcription can be positively and negatively regulated. Based on studies of bacterial repressors, the concept of steric hindrance, whereby RNA polymerase is denied access to the promoter because of repressor binding, has originally dominated all models of gene regulation. When eukaryotic genes were studied in more detail, it became apparent that

positive regulation of gene activity is widespread. Unlike the situation in bacteria, eukaryotic RNA polymerase II cannot recognize a promoter by itself, but has to be guided to the initiation site by DNA-bound transactivators (Mitchell and Tjian, 1989). This is probably due to the fact that DNA is packaged into nucleosomes, hence a nonspecific transcriptional repression may represent the default state of eukaryotic gene expression (Svaren and Hörz, 1996; however, see also Beato *et al.*, 1996). Even though it might, in principle, be sufficient to have eukaryotic transcription entirely regulated by an antagonism of histones versus activator proteins, the situation is much more complex. There is, e.g., evidence for different levels of chromatin structure: DNA methylation, a low degree of histone acetylation, and high levels of histone H1 are correlated with a compact chromatin structure that can cover large chromosome segments. This so-called heterochromatin seems to ensure that the background level of transcription is reduced to a minimum (for review, see e.g. Edmondson *et al.*, 1996; Felsenfeld, 1996; Roth and Allis, 1996; Stargell and Struhl, 1996; Vanholde and Zlatanova, 1996).

Some of the repressor/corepressor proteins of yeast, *Drosophila* and mammals act by interfering with the process of transcriptional initiation (Hanna-Rose and Hansen, 1996), whereas the so-called Polycomb-group proteins, which are required for maintenance of the repressed state of the homeotic genes in *Drosophila* development and also are present in mammalian cells, most likely function by inducing the packaging of their target genes into transcriptionally inactive heterochromatin (e.g., Zink and Paro, 1995; Strutt *et al.*, 1997). The term transcriptional silencing is often used to describe repression effects in eukaryotes, notably repression over long distances as first described for the yeast mating type locus by K. Nasmyth and colleagues (Brand *et al.*, 1985).

Eukaryotic repressors may principally act in three different ways:

- (i) By steric hindrance, similar to bacterial repressors;
- (ii) by specifically interfering with the assembly of a functional transcription initiation complex ('active repression'; for review, see Hanna-Rose and Hansen, 1996); or
- (iii) by nucleosome remodeling, e.g. by inducing changes in the state of histone acetylation and/or recruiting proteins that participate in establishment and maintenance of an inaccessible chromatin structure (reviewed, e.g., in Kingston *et al.*, 1996).

Transcriptional silencing could in principle be brought about by mechanisms (ii) and (iii).

About one third of the human zinc finger proteins of the Krüppel type (Cys2 His2) contain at their N-terminus a conserved domain of about 75 aa, which is referred to as the Krüppel-associated box (KRAB) domain (Bellefroid *et al.*, 1991). It does not occur in yeast and probably not in *Drosophila*, suggesting its late appearance in evolution. The distinct spatial and temporal expression pattern of several KRAB domain-containing zinc finger factors may indicate an important role in cell differentiation and development, however, their actual function remains to be elucidated (Bellefroid *et al.*, 1991, 1993; Witzgall *et al.*, 1993). The KRAB domain of the human zinc finger proteins KOX1 and Kid-1 have been most extensively studied so far. When tethered to DNA, it confers a negative effect on gene transcription, which was demonstrated by fusing it to the heterologous DNA binding domain (DBD) of GAL4 (Margolin *et al.*, 1994; Witzgall *et al.*, 1993 and 1994). It was found that the GAL4-KRAB fusion can silence transcription in a dose-dependent manner and over a long distance (Pengue *et al.*, 1994; Deuschle *et al.*, 1995). The strong repression potential of the KRAB domain has led to a search for its cellular partners. Several groups, including ours, independently found that the KRAB domain specifically in-

teracts with a nuclear protein, TIF1 β (also referred to as KAP1 or KRIP-1), which shows several structural features of chromatin-associated proteins (Friedman *et al.*, 1996; Le Douarin *et al.*, 1996; Moosmann *et al.*, 1996; Kim *et al.*, 1996). TIF1 β , in turn, interacts with the mammalian heterochromatin-associated factors HP1 α , MOD1 and MOD2 (Koonin *et al.*, 1995; Le Douarin *et al.*, 1996). These findings suggested that the KRAB domain exerts its repression via influencing the chromatin structure.

In this study we have refined the analysis of position effects of KRAB-mediated repression, and demonstrate a broad activity of the KRAB domain: it is able to repress transcription that is driven by several different activation domains fused to GAL4 and by a whole activator protein. Most remarkably, transcription of an RNA polymerase III-dependent gene is also repressed, whereas transcription by RNA polymerase I and by phage T7 RNA polymerase are refractory to KRAB-mediated repression. These latter findings argue against a general, physical inaccessibility of template DNA induced by the KRAB domain but are reminiscent of the action of the DR1/DRAP1 repressor which represses RNA pol II and III but not pol I transcription (White *et al.*, 1994; Kim *et al.*, 1997).

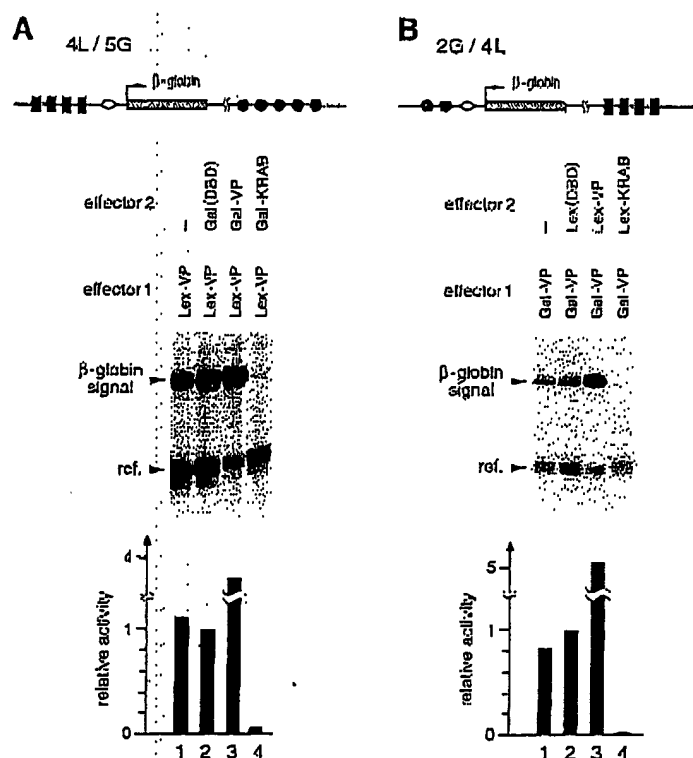


Fig. 1 Repression over Long Distances also Works with a KRAB-LexA Fusion Protein.

Two reporter genes were tested in transiently transfected HeLa cells, one with LexA upstream or downstream of GAL4 sites (A) and (B), respectively. The DNA binding domains (DBD) of LexA and GAL4 factors were fused to the VP16 activation domain, or to the KRAB domain. Combinations of chimeric factors were coexpressed as indicated. Strong activation resulted from coexpression of both Lex-VP and GAL-VP (A and B, lanes 3), the DBDs of LexA and GAL4 did not contribute to activation (A and B, lanes 2). KRAB abrogated transcription in both constellations (A and B, lane 4). Bar diagrams show expression levels of the reporter gene (β -globin signal) normalized to expression levels of a cotransfected reference gene (Westin *et al.*, 1987).

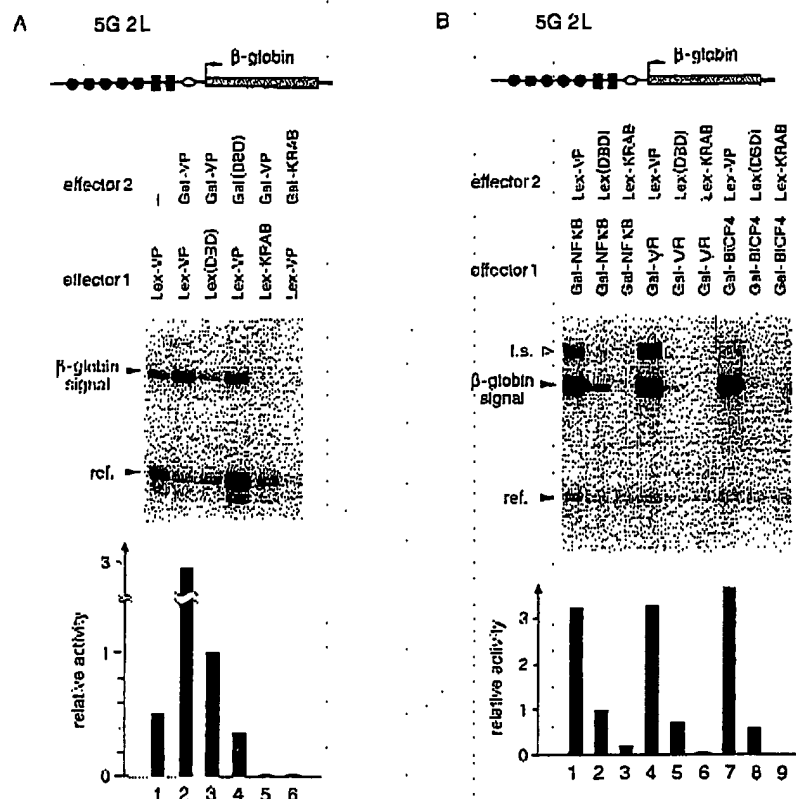


Fig. 2 KRAB Domain Represses Transcription Driven by Different Activators.

(A) KRAB represses in two different promoter-proximal positions. While Lex-VP and GAL-VP activated synergistically (lane 2), both KRAB fusions repressed transcription from a reporter construct containing five GAL4 upstream of two LexA sites (lanes 5 and 6 vs. lanes 3 and 4). (B) In the same way, the artificial activators composed of GAL4 DNA binding domain and the activation domains of NF κ B (RelA/p55 subunit), pseudorabies (PRVIE) immediate early and the bovine herpes virus immediate early protein (BICP4), respectively, were repressed in their function (lanes 3, 6, 9 vs. 2, 5 and 8). A strong synergism was observed in combination with Lex-VP16 (lanes 1, 4, and 7), whereas Lex(DBD) behaved neutral (lanes 2, 5 and 8 and data not shown). As in (A), the reporter gene contained five GAL4 sites upstream of two LexA sites, and was tested under coexpression of LexA fusion proteins, as indicated. Transcripts starting further upstream, presumably at a pseudo-TATA box within the LexA recognition sites, are indicated with f.s. ('false start').

Results

The KRAB Domain Silences Transcription over Large Distances, Independently of DNA Binding Site/Binding Domain

We have tested the KRAB effect on synthetic promoters of the structure 4xLexA / reporter gene / 5xGAL4. Promoter-bound Lex-VP16 activator strongly activated transcription (Figure 1A) and its activity was further boosted by concomitant activation from a downstream position via GAL-VP16 factor. In this downstream position the GAL4 DNA binding domain (DBD) behaved in a neutral manner, i.e., as if no GAL4 fusion factor was added at all, whereas Lex-VP16 activation from the promoter position was strongly reduced (down to 1/8th) by binding of GAL-KRAB in the remote enhancer position. The converse experiment, with two upstream GAL4 and four downstream LexA binding sites, yielded essentially the same result (Figure 1B): activation from the promoter position exerted by GAL-VP16 was effectively blocked by enhancer-bound Lex-KRAB. Similar to the situation with GAL4 factors, Lex-VP16 activated further, whereas the Lex DBD behaved neutral from this remote position.

Transcription under Control of Several Activator Domains Is Repressed

In a configuration: binding site X / binding site Y / TATA box, whereby X and Y were GAL4 and LexA binding sites, or vice versa, the KRAB domain inhibits transcription when tethered to DNA either upstream or downstream of the activating factor. In this experiment, five GAL4 sites followed by two LexA sites upstream of the TATA box were tested with the entire collection of LexA and GAL4 fusions (Figure 2A). The results show that in both cases the KRAB domain can effectively block transcription driven by nearby activating factors, irrespective of whether the KRAB domain was fused to LexA or to GAL4 DBD (Figure 2A, lanes 5 and 6, respectively).

In further experiments we have shown that the transcriptional repression is general, rather than specifically depending on the herpes simplex virus VP16 activator. As is seen in Figure 2B, we have tested further activation domains fused to GAL4, namely the activation domain derived from the C-terminus of NF κ B p55 protein, and the strong acidic activation domains of immediate early proteins of pseudorabies (GAL-PRVIE) or bovine herpes virus (GAL-BICP4) (Gstaiger *et al.*, 1994). All these activators

strongly boosted transcription, an effect further reinforced by the presence of Lex-VP16 fusion protein. The Lex DBD gave an intermediary level of transcription, while Lex-KRAB abrogated transcription stimulated by these activators.

Transcription Boosted by the Pseudorabies Virus Immediate Early Coactivator Is Inhibited by the KRAB Domain

In order to see whether the KRAB domain could exert the negative effect also on an entire activating protein, rather than chimeric proteins of the GAL and Lex type, we chose the major immediate early protein of pseudorabies virus, which is a very strong coactivator protein. As we have shown before, this immediate early protein is not only active on viral promoters but boosts the activity of a large collection of synthetic promoters that are driven by cellular activators (Thali et al., 1990 and refs. therein). PRVIE indeed activated transcription of an Sp1 site promoter to a high level (Figure 3). The presence of GAL-KRAB binding in the downstream enhancer position reduced this level to about 1/7th. As observed in previous experiments, the GAL4 DBD had neither a positive nor a negative effect on transcription driven by Sp1 and PRV immediate early protein.

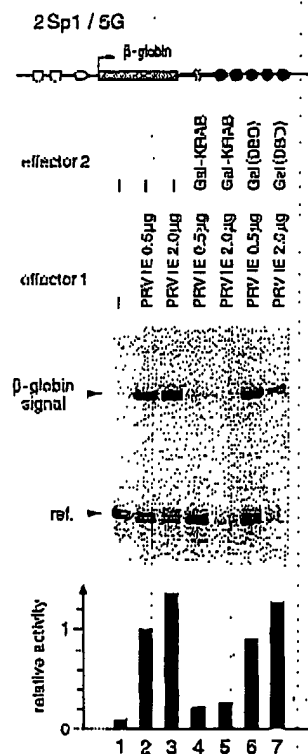


Fig. 3 The KRAB Domain Inhibits Transcriptional Activation by a General Herpes Viral Activator.

Pseudorabies virus IE protein activates a minimal promoter consisting of a TATA box and Sp1 sites (lanes 2 and 3 vs. lane 1). Transcription is inhibited upon concomitant expression of a chimeric protein containing the KRAB domain tethered to multiple GAL4 sites downstream of the β -globin reporter gene (lanes 4 and 5), while no inhibition is seen when only the GAL4 DBD is coexpressed (lanes 6 and 7).

The KRAB Domain also Represses RNA Polymerase III Transcription

In yeast, mating type silencing has been shown to affect both RNA polymerase II and III transcription (Brand et al., 1985; Schnell and Rine, 1986), while the corepressor complex TUP1/SSN6 is known to repress transcription by RNA polymerase I and II, but not that by RNA polymerase III (Herschbach and Johnson, 1993). Therefore, we chose the adenovirus VA RNA I gene to test whether the KRAB domain would affect RNA polymerase III driven transcription. In our standard experiments with RNA polymerase II, we had provided both the activator and the repressor from transfected expression plasmids. However, in these RNA polymerase III experiments, only the KRAB repressor was transfected, while activation relied entirely on endogenous RNA polymerase III transcription factors. Under these conditions, activation had a headstart while the cell still had to produce enough GAL-KRAB protein for any repression effect to manifest itself. Furthermore, as had been demonstrated *in vitro*, RNA polymerase III can stay on the same template DNA for multiple rounds of transcription (Diaci and Sentenac, 1996), which would hinder any repression mechanism that would interfere with RNA polymerase recruitment. Indeed, the KRAB domain was also able to repress the VA RNA I transcription from binding sites both upstream and approximately 1.8 kb downstream of the initiation site, as shown in Figure 4. In the

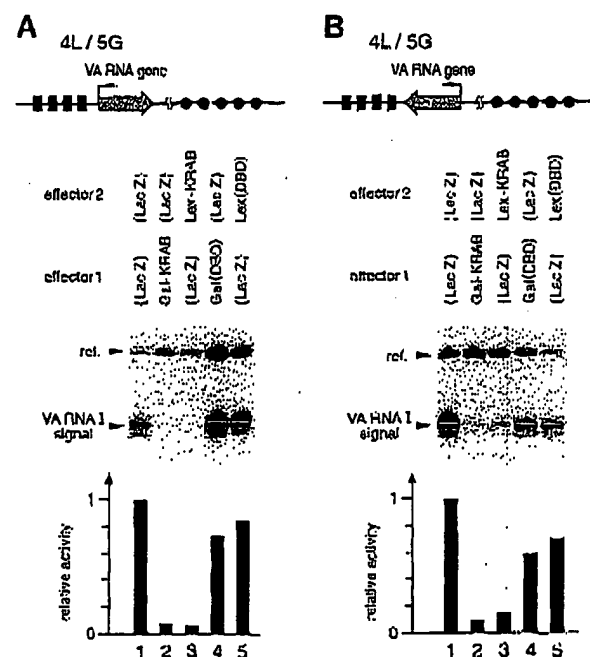


Fig. 4 The KRAB Domain Represses RNA Polymerase III-Dependent Transcription of the Adenovirus VA RNA I Gene.

The reporter gene was cloned in both orientations into a vector containing 4 LexA and 5 GAL4 binding sites near to and far from the insertion site, respectively. From both positions, the KRAB domain exerted a repression effect on the VA RNA I gene (e.g., lanes A2 vs. A3). A plasmid encoding β -galactosidase (LacZ) was used as a control.

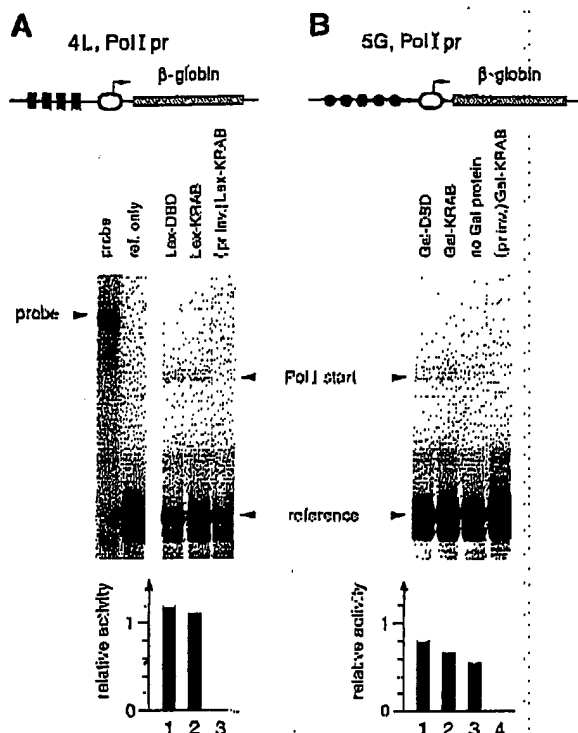


Fig. 5 RNA Polymerase I-Dependent Transcription Is Refractory to Repression by the KRAB Domain.

A DNA segment containing the human rRNA promoter (white oval) was inserted upstream of two β -globin reporter genes that also contained binding sites for either LexA repressor (A) or GAL4 protein (B) (for details see Materials and Methods). Human 293 cells were transfected with these reporter genes, and transcript levels determined by the S1 nuclease protection assay, as before. Transcripts were determined in the presence of LexA or GAL4 DNA-binding domain (DBD) (lanes 1), in the presence of LexA-KRAB or GAL-KRAB fusion proteins (lanes 2), in absence of any GAL protein (B, lane 3) or with GAL-KRAB fusion but using a reporter gene with inverted RNA polymerase I promoter segment (A, lane 3 and B, lane 4).

light of the arguments mentioned above, we observed that repression by the KRAB domain was moderate at shorter times and became more obvious after long (48 h) incubation times (Figure 4 and data not shown).

Transcription Directed by RNA Polymerase I or by Phage T7 RNA Polymerase in Mammalian Cells Is Not Inhibited by the KRAB Domain

The results with RNA polymerases II and III could mean that the KRAB domain acted via a specific mechanism, such as a cofactor or subunit shared by both polymerases, or by a more general mechanism. To find out whether RNA polymerase I, which is responsible for rRNA gene transcription, or a heterologous phage RNA polymerase could be inhibited by the KRAB domain, we inserted the appropriate promoters for RNA polymerase I and for the small, single subunit RNA polymerase of bacteriophage T7, upstream of β -globin reporter genes that also contained binding sites for either LexA repressor or GAL4 protein. While RNA polymerase I shares some subunits and at

least one cofactor (TBP) with the other two RNA polymerases, the T7 RNA polymerase and its cognate promoter should represent a completely heterologous system. For this reason, the latter has been used before as a model system in eukaryotic cells and cell extracts (e.g., Chen *et al.*, 1987; Fuerst *et al.*, 1986; Jenuwein *et al.*, 1997; McCall and Bender, 1996; see Discussion). Curiously, we obtained only very weak signals in HeLa cells, indicating instability of the transcripts of both Pol I and T7 RNA polymerase, perhaps as a result of the missing cap structure (not shown; see also Smale and Tjian, 1985 and Kuhn *et al.*, 1990). Fortunately, the problem was overcome by transfection into human 293 cells. These cells behave normally with respect to KRAB silencing of RNA polymerase II templates (not shown). We transfected 293 cells with reporter genes dependent on either RNA polymerase I or T7 RNA polymerase, and quantified transcripts two days later by S1 nuclease. In neither case was there any substantial influence of the KRAB domain on transcription (Figures 5 and 6), unlike the findings with RNA polymerases II and III.

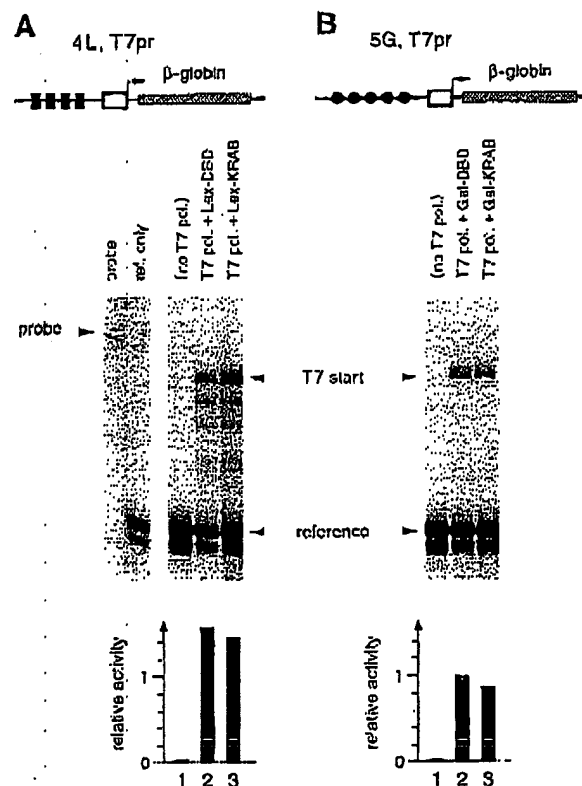


Fig. 6 The KRAB Domain Fails to Inhibit Transcription by Phage T7 RNA Polymerase in Mammalian Cells.

A promoter for T7 RNA polymerase (white box) was inserted upstream of two β -globin reporter genes that also contained binding sites for either LexA repressor (A) or GAL4 protein (B). Human 293 cells were transfected with these reporter genes, and transcript levels determined. Lanes 1, transcripts in absence of an expression vector encoding T7 polymerase (negative control). Lanes 2, response to T7 RNA polymerase, in the presence of LexA or GAL4 DNA-binding domain (DBD). Lanes 3, response to T7 polymerase in the presence of LexA-KRAB or GAL-KRAB fusion proteins. In an independent experiment, the same transcript level was also observed in the absence of any LexA or GAL4 chimeras (not shown).

Discussion

In its natural context, the KRAB domain of the KRX1/ZNF10 zinc finger protein is located near the N-terminus. Similarly, about one third of all mammalian zinc finger proteins of the Krüppel type (Cys2 His2) harbour a KRAB-related domain in their N-terminal region. When tethered to DNA via a heterologous DNA binding domain, this protein domain strongly inhibits transcription from a nearby promoter. In our experiments, binding to DNA was achieved via either LexA repressor or GAL4, which contain DNA binding domains of the helix-turn-helix and zinc cluster type, respectively. This indicates that the KRAB domain is a general repressing domain, rather than being dependent on the greater protein context of a specific type of zinc finger factor.

The inhibition of transcription by the KRAB domain was a *cis*-effect; expression of the reference gene, for example, was not affected in any way by the presence of a large amount of KRAB domain in the cell (see also Moosmann *et al.*, 1996). We find it particularly interesting that the KRAB domain can efficiently silence transcription from a remote position at 1.8 kb downstream of the site of transcription initiation, downstream of the reporter gene (Figure 2). Silencing at a distance was also found by others (Pengue *et al.*, 1994; Deuschle *et al.*, 1995), however without mapping of the transcriptional start sites, which we consider necessary to rule out effects of read through transcription on a circular plasmid. We have shown in our study that the KRAB domain can generally repress transcription, using different activation domains and distances to the initiation site. In addition, we have tested transcription by three other RNA polymerases, namely RNA pol I, pol III and phage T7 polymerase. The data show that the KRAB domain does not repress any kind of transcription. Rather, it appears to interfere with proteins essential for transcription by RNA polymerases II and III and/or affect chromatin structure in a selective manner.

Therefore, an important question concerns the cellular partners of the KRAB domain. We and others have used a yeast system to select for KRAB-interacting proteins. One of these was identified as TIF1 β , a protein of the RBCC (RING finger, B box, coiled coil) family. TIF1 β , like KRAB itself, also represses transcription in transient transfections when tethered to template DNA (Friedman *et al.*, 1996; Le Douarin *et al.*, 1996; Moosmann *et al.*, 1996; Kim *et al.*, 1996). Interestingly, TIF1 β was isolated by the group of R. Losson and P. Chambon by virtue of its interaction with heterochromatin-enriched proteins HP1 α , MOD1 and MOD2 (Le Douarin *et al.*, 1996). This connection, taken at face value, strongly favors a KRAB silencing mechanism via chromatin remodeling. However, the results with RNA polymerase I and with T7 RNA polymerase should caution us against a straightforward interpretation: Neither Lex-KRAB nor GAL-KRAB fusion proteins were able to significantly affect transcription from an RNA pol I or T7 promoter. The combination T7 promoter/T7 polymerase has been used before in eukaryotes, as a heterologous system

to probe for physical accessibility of template DNA. However, these previous studies have yielded seemingly contradictory results. Recently it was found that chromatin extracted from inactive immunoglobulin genes strongly inhibited transcription from a built-in T7 promoter

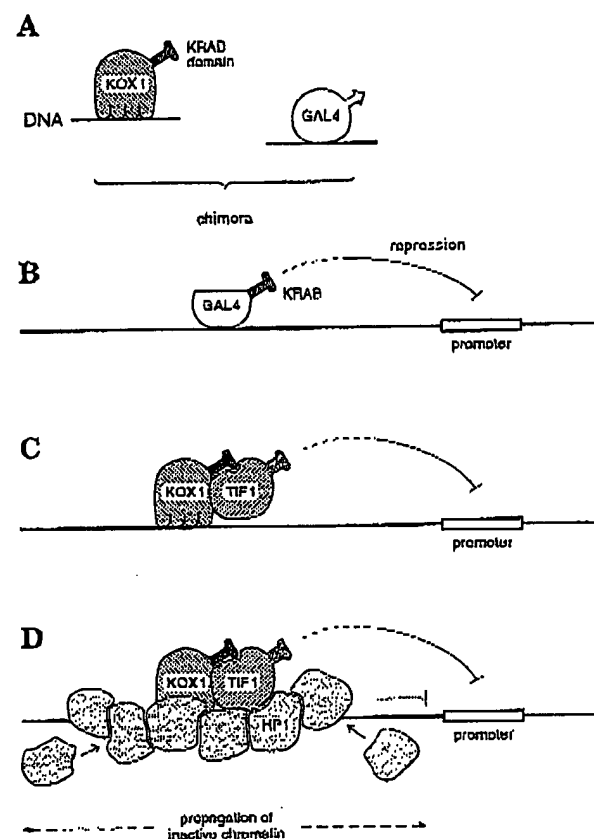


Fig. 7 Model of KRAB Repression Mechanism(s).

(A, B) The KRAB domain, which is naturally part of a zinc finger factor, such as KRX1 or Kid-1, can be tethered to DNA via heterologous binding sites. It then efficiently represses a promoter in *cis* even over large distances. (C) The KRAB domain interacts with TIF1 β (also termed KAP1 or KRIP1, see text) and TIF1 α proteins. Both TIF1 α and β are members of the RBCC (RING finger, B box, coiled coil) family which by themselves can repress transcription when tethered to DNA, as shown under (B) (Le Douarin *et al.*, 1996; Moosmann *et al.*, 1996). In this model, the zinc finger protein KRX1 is assumed to be a DNA-binding protein. However, zinc fingers can also be implicated in RNA binding, and recent data involve specific RNAs in transcriptional silencing (Herzing *et al.*, 1997; Lee and Jaenisch, 1997 and references therein). (D) TIF1 (α or β) factors bind heterochromatin-enriched components such as HP1 α , MOD1 and MOD2 with a region neither involving their KRAB interaction domain nor their own repression domain (Le Douarin *et al.*, 1996). TIF1 α and β may thus induce the formation of inactive chromatin, perhaps in addition to an 'active repression' mechanism involving components of the transcription apparatus. It is presently not known whether the interaction of the KRAB domain with TIF1 proteins is a prerequisite for KRAB-mediated silencing. Also, the potential interplay of KRAB/TIF proteins with nucleosomal structure, not indicated in this model but thought to be crucial for inactive chromatin formation, remains to be investigated.

(Jenuwein *et al.*, 1997). By contrast, using a semiquantitative staining assay of whole *Drosophila* embryos, heterochromatin organized by Polycomb protein did not hamper expression of a β -galactosidase transgene driven by T7 RNA polymerase (McCall and Bender, 1996). As a positive control, in the same system, a strong repression effect was found on activation by GAL4 factor (see also Zink and Paro, 1995). However, even within *Drosophila*, there are qualitative differences between telomeric and pericentric heterochromatin (Wallrath and Elgin, 1995). This indicates that not all types of inactive chromatin are alike, and could reconcile the seemingly conflicting results obtained so far. In any case, it seems possible that transcriptionally inactive chromatin structures are exerting specific inhibitory effects towards one or the other RNA polymerase, rather than mounting a physical barrier that would generally inhibit access to template DNA.

In this context it seems worth mentioning that the DR1/DRAP1 repressor that is conserved from yeast to man has a similar activity spectrum as the KRAB domain described in here, in that it blocks transcription by RNA polymerases II and III but not polymerase I (White *et al.*, 1994; Kim *et al.*, 1997). DR1 binds to the TATA-binding protein TBP and blocks the interaction of TBP with polymerase II- and polymerase III-specific factors. Even though TBP is an essential cofactor for transcription by all three polymerases, the activity of RNA polymerase I is apparently not affected by DR1. However, the KRAB domain studied here is neither found in yeast proteins, nor do GAL-KRAB fusions silence yeast transcription, which indicates to us that the relation of KRAB to DR1, if any, would have evolved late in evolution.

All data from different laboratories taken together, the KRAB domain probably exerts a specific, active repression effect on both RNA polymerases II and III, and in addition it may also help to organize, under appropriate conditions, an inactive chromatin structure via interaction with TIF1 β and the 'chromo shadow' domain proteins MOD1, MOD2, and HP1 α , as depicted in the model of Figure 7.

Materials and Methods

Recombinant DNA work was done according to standard protocols. Details concerning construction of plasmids, which were verified by sequencing, are available upon request (E-mail: Oleg Georgiev<ole@molbio2.unizh.ch>).

Plasmid Construction

The reporter gene constructs are based on the plasmid OVEC (Westin *et al.*, 1987). They contain the respective binding sites either in upstream-'promoter' or in downstream-'enhancer' position (Hug *et al.*, 1996; Moosmann *et al.*, 1996; Seipel *et al.*, 1992). To construct pVA-OVEC, a 480 base pair *Hind* III fragment containing the VA RNA I gene (Svensson and Akusjärvi, 1994) was cloned into the *Pst* I site of 4L/5G OVEC and analyzed as described in Gerber *et al.* (1995). For the experiments with the T7 RNA polymerase, a 30 nt fragment comprising the T7 promoter was cloned into OVEC vector that also contained the binding

sites for the respective regulatory factor. The mammalian T7 RNA polymerase expression vector was a kind gift of Dr. M. Billeter, Zürich. In the case of RNA polymerase I-dependent reporter constructs, human spleen genomic DNA was amplified with rDNA-specific primers and the PCR products (-252 to +29 and -252 to +87) were cloned into the *Pst* I site of 4L/5G OVEC.

Transfection and RNA Analysis

HeLa cells were grown under standard conditions and transfected by the calcium phosphate coprecipitation method (Westin *et al.*, 1987) with 5 μ g of OVEC reporter plasmid, 3 μ g of transactivator plasmid and 1.5 μ g reference plasmid. The total amount of DNA transfected was adjusted with empty vector plasmid to 20 μ g per 10 cm dish. OVEC-REF was used for reference with exception of the experiments with the VA RNA I gene, where a constitutively active OVEC construct was used. After 36 hours of incubation, RNA was isolated according to Schreiber *et al.* (1988), and hybridized to a radiolabeled oligonucleotide (Westin *et al.*, 1987). Hybridization was performed overnight at 30 °C. Hybridization products were digested with 150U S1 nuclease for 1 hour and separated on a 10% denaturing polyacrylamide gel. For quantification, dried gels were exposed to a phosphor storage screen or autoradiographs were analyzed densitometrically (Molecular Dynamics, Inc.). The signals derived from the reference transcripts were used to normalize for variability in the transfection efficiency. Probably due to instability of uncapped transcripts, only very weak signals were detected with RNA polymerase I and T7 RNA polymerase in HeLa cells, while in 293 cells transcripts could be readily detected.

Note Added in Proof

We have verified the transcript bands marked in Figure 5 as 'Pol I start' to represent genuine Pol I transcripts by virtue of their insensitivity to 2.5 μ g/ml and even 25 μ g/ml α -amanitin, conditions under which the Pol II transcript of the reference gene has been eliminated.

Acknowledgements

We are indebted to Dr. Antonia Manova and René Peclard for technical help, to Drs. Martin Billeter (Zürich), Ingrid Grummt and Renate Voil (Heidelberg) for gifts of T7 polymerase and Pol I promoter plasmids, to Dr. Joy Alcedo for critical reading of the manuscript and to Fritz Ochsenbein for excellent artwork.

References

- Beato, M., Chavez, S., and Truss, M. (1996). Transcriptional regulation by steroid hormones. *Steroids* 61, 240–251.
- Bellefroid, E.J., Poncelet, D.A., Lecocq, P.J., Revelant, O., and Martial, J.A. (1991). The evolutionarily conserved Krüppel-associated box domain defines a subfamily of eukaryotic multi-fingered proteins. *Proc. Natl. Acad. Sci. USA* 88, 3608–3612.
- Bellefroid, E.J., Marine, J.C., Ried, T., Lecocq, P.J., Rivière, M., Amarniya, C., Poncelet, D.A., Coulie, P.G., de, J.P., Szpirer, C. *et al.* (1993). Clustered organization of homologous KRAB zinc finger genes with enhanced expression in human T lymphoid cells. *EMBO J.* 12, 1363–1374.
- Brand, A.H., Breeden, L., Abraham, J., Sternglanz, R., and Nasmyth, K. (1985). Characterization of a 'silencer' in yeast: a DNA

- sequence with properties opposite to those of a transcriptional enhancer. *Cell* 41, 41–48.
- Chen, W., Tabor, S., and Struhl, K. (1987). Distinguishing between mechanisms of eukaryotic transcriptional activation with bacteriophage T7 RNA polymerase. *Cell* 50, 1047–1055.
- Deuschle, U., Moyer, W.K.-H., and Thiesen, H. J. (1995). Tetracycline-reversible silencing of eukaryotic promoters. *Mol. Cell. Biol.* 15, 1907–1914.
- Dieci, G., and Sentenac, A. (1996). Facilitated recycling pathway for RNA polymerase III. *Cell* 84, 245–252.
- Edmondson, D.G., Smith, M.M., and Roth, S.Y. (1996). Repression domain of the yeast global repressor TUP1 interacts directly with histones H3 and H4. *Genes Dev.* 10, 1247–1259.
- Felsenfeld, G. (1996). Chromatin unfolds. *Cell* 86, 13–19.
- Friedman, J.R., Fredericks, W.J., Jensen, D.E., Speicher, D.W., Huang, X.-P., Neilson, E.G., and Tauscher III, F.J. (1996). KAP-1, a novel corepressor of the highly conserved KRAB repression domain. *Genes Dev.* 10, 2067–2078.
- Fuersat, T.R., Niles, E.G., Studier, F.W., and Moss, B. (1986). Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc. Natl. Acad. Sci. USA* 83, 8122–8126.
- Gerber, H.P., Hagmann, M., Seipel, K., Georgiev, O., West, M.A., Litting, Y., Schaffner, W., and Corden, J.L. (1995). RNA polymerase II C-terminal domain required for enhancer-driven transcription. *Nature* 374, 660–662.
- Gstaiger, M., and Schaffner, W. (1994). Strong transcriptional activators isolated from viral DNA by the 'activator trap', a novel selection system in mammalian cells. *Nucl. Acids Res.* 22, 4031–4038.
- Hanna-Rose, W., and Hansen, U. (1996). Active repression mechanisms of eukaryotic transcription repressors. *Trends Genet.* 12, 229–234.
- Herschbach, B.M., and Johnson, A.D. (1993). The yeast $\alpha 2$ protein can repress transcription by RNA polymerases I and II but not III. *Molec. Cell. Biol.* 13, 4029–4038.
- Horzing, L.B.K., Rorner, J.T., Horn, J.M., and Ashworth, A. (1997). Xist has properties of the X-chromosome inactivation centre. *Nature* 386, 272–275.
- Hug, M., Silke, J., Georgiev, O., Rusconi, S., Schaffner, W., and Matsuo, K. (1996). Transcriptional repression by methylation: cooperativity between a CpG cluster in the promoter and remote CpG-rich regions. *FEBS Lett.* 379, 251–254.
- Jenuwein, T., Forrester, W.C., Fernandezherero, L.A., Laible, G., Dull, M., and Grosschedl, R. (1997). Extension of chromatin accessibility by nuclear matrix attachment regions. *Nature* 385, 269–272.
- Kim, S.S., Chen, Y.M., O'Leary, E., Witzgall, R., Vidal, M., and Bonventre, J.V. (1996). A novel member of the RING finger family, KRIP-1, associates with the K1AB-A transcriptional repressor domain of zinc finger proteins. *Proc. Natl. Acad. Sci. USA* 93, 15299–15304.
- Kim, S., Na, J.G., Hampsey, M., and Roiberg, D. (1997). The DR1/DRAP1 heterodimer is a global repressor of transcription *in vivo*. *Proc. Natl. Acad. Sci. USA* 94, 820–825.
- Kingston, R.E., Bunker, C.A., and Imbalzano, A.N. (1996). Repression and activation by multiprotein complexes that alter chromatin structure. *Genes Dev.* 10, 905–920.
- Koonin, E.V., Zhou, S., and Lucchesi, J.C. (1995). The chromo superfamily: new members, duplication of the chromo domain and possible role in delivering transcription regulators to chromatin. *Nucleic Acids Res.* 23, 4229–4233.
- Kuhn, A., Deppert, U., and Grummt, I. (1990). A 140-base-pair repetitive sequence element in the mouse rRNA gene spacer enhances transcription by RNA polymerase I in a cell-free system. *Proc. Natl. Acad. Sci. USA* 87, 7527–7531.
- Le Douarin, B., Nielsen, A.L., Garnier, J.-M., Ichinose, H., Jeannouin, F., Losson, R., and Chambon, P. (1996). A possible involvement of TIF1 α and TIF1 β in epigenetic control of transcription by nuclear receptors. *EMBO J.* 15, 6701–6715.
- Lee, J.T., and Jaenisch, R. (1997). Long range cis effects of ectopic X-inactivation centres on a mouse autosome. *Nature* 386, 375–379.
- Margolin, J.F., Friedman, J.R., Meyer, W.K., Vissing, H., Thiesen, H.J., and Rauscher, F.R. (1994). Krüppel-associated boxes are potent transcriptional repression domains. *Proc. Natl. Acad. Sci. USA* 91, 4509–4513.
- McCall, K., and Bender, W. (1996). Probes of chromatin accessibility in the *Drosophila* bithorax complex respond differently to Polycomb-mediated repression. *EMBO J.* 15, 569–580.
- Mitchell, P.J., and Tjian, R. (1989). Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science* 245, 371–378.
- Moosmann, P., Georgiev, O., Le Douarin, B., Bourquin, J.P., and Schaffner, W. (1996). Transcriptional repression by RING finger protein TIF1 β that interacts with the KRAB repressor domain of KOX1. *Nucleic Acids Res.* 24, 4859–4867.
- Penque, G., Calabro, V., Bartoli, P.C., Pagliuca, A., and Lania, L. (1994). Repression of transcriptional activity at a distance by the evolutionarily conserved KRAB domain present in a subfamily of zinc finger proteins. *Nucleic Acids Res.* 22, 2908–2914.
- Roth, S.Y., and Allis, C.D. (1996). Histone acetylation chromatin assembly—a single escort, multiple dances. *Cell* 87, 5–8.
- Schnell, R., and Rine, J. (1986). A position effect on the expression of a tRNA gene mediated by the *SIR* genes in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 6, 494–501.
- Schreiber, E., Matthias, P., Mueller, M.M., and Schaffner, W. (1988). Identification of a novel lymphoid specific octamer binding protein OTF-2 β by proteolytic clipping bandshift assay PCBA. *EMBO J.* 7, 4221–4230.
- Seipel, K., Georgiev, O., and Schaffner, W. (1992). Different activation domains stimulate transcription from remote ('enhancer') and proximal ('promoter') positions. *EMBO J.* 11, 4961–4968.
- Smale, S.T., and Tjian, R. (1985). Transcription of Herpes Simplex Virus tk Sequences Under the Control of Wild-Type and Mutant Human RNA Polymerase I Promoters. *Mol. Cell. Biol.* 5, 352–362.
- Stargell, L.A., and Struhl, K. (1996). Mechanisms of transcriptional activation *in vivo*—two steps forward. *Trends Genet.* 12, 311–315.
- Strutt, H., Cavalli, G., and Paro, R. (1997). Co-localization of Polycomb protein and GAGA factor on regulatory elements responsible for the maintenance of homeotic gene expression. *EMBO J.* 16, in press.
- Svaren, J., and Hörz, W. (1996). Regulation of gene expression by nucleosomes. *Curr. Opin. Genet. Dev.* 6, 164–170.
- Svensson, C., and Akusjärvi, G. (1984). Adenovirus VA RNA1: a positive regulator of mRNA translation. *Mol. Cell. Biol.* 4, 736–742.
- Thali, M., Rusconi, S., and Schaffner, W. (1990). Immediate early protein of pseudorabies virus is a general transactivator but stimulates only suboptimally utilized promoters. A clue to specificity? *J. Molec. Biol.* 215, 301–311.
- Vanhilde, K., and Zlatanova, J. (1996). What determines the folding of the chromatin fiber. *Proc. Natl. Acad. Sci. USA* 93, 10548–10555.
- Wallrath, L.L., and Elgin, S.C. (1995). Position effect variegation in *Drosophila* is associated with an altered chromatin structure. *Genes Dev.* 9, 1263–1277.

- Westin, G., Gerster, T., Muller, M.M., Schaffner, G., and Schaffner, W. (1987). OVEC, a versatile system to study transcription in mammalian cells and cell-free extracts. *Nucleic Acids Res.* 15, 6787-6798.
- White, R.J., Khoo, B.C., Inostroza, J.A., Reinberg, D., and Jackson, S.P. (1994). Differential regulation of RNA polymerase I, II, and III by the TBP-binding repressor DR1. *Science* 264, 418-450.
- Witzgall, R., O'Leary, E., Gessner, R., Ouellette, A.J., and Bonventre, J.V. (1993). Kid-1, a putative renal transcription factor: regulation during ontogeny and in response to ischemia and toxic injury. *Molec. Cell. Biol.* 13, 1933-1942.
- Witzgall, R., O'Leary, E., Lear, A., Onaldi, D., and Bonventre, J.V. (1994). The Krüppel-associated box-A (KRAB-A) domain of zinc finger proteins mediates transcriptional repression. *Proc. Natl. Acad. Sci. USA* 91, 4514-4518.
- Zink, D., and Paro, R. (1995). *Drosophila* Polycomb-group regulated chromatin inhibits the accessibility of a transactivator to its target DNA. *EMBO J.* 14, 5660-5671.

Received May 5, 1997; accepted May 16, 1997